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## Response of bacterioplankton to iron fertilization in the Southern Ocean

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### Abstract

We studied the bacterial response to Fe fertilization over 3 weeks during the second iron-enrichment experiment (EisenEx) in the Southern Ocean. Bacterial abundance in the Fe-fertilized patch increased over the first 12 d following Fe release and remained about twice as high as outside the Fe-fertilized patch until the end of the experiment. Bacterial production peaked a few days after each of the three Fe releases inside the Fe-fertilized patch, reaching rates two to three times higher than outside the patch. Besides the peaks in leucine and thymidine incorporation following Fe release, bacterial production was not significantly higher inside the patch than outside, suggesting direct limitation of bacterial growth by Fe. Bacterial aminopeptidase activity roughly followed the increase in bacterial abundance, whereas cell-specific  $\alpha$ - and  $\beta$ -glucosidase were higher inside the Fe-fertilized patch. The diversity of  $\beta$ -glucosidases was determined by capillary electrophoresis zymography. The different  $\beta$ -glucosidases showed much higher activity levels inside the patch than in the surrounding waters, and three additional  $\beta$ -glucosidases constituting ~55% of the total  $\beta$ -glucosidase activity were present inside the Fe-fertilized patch from day 9 onward. No major changes in response to Fe fertilization were detected in the phylogenetic composition of the bacterioplankton community, as determined by 16S rDNA fingerprinting, indicating a remarkable adaptation of the bacterioplankton community to episodic iron inputs. This stability on the phylogenetic level is contrasted by the dramatic qualitative and quantitative changes in ectoenzymatic activity.

Bacterioplankton play a central role in the nutrient and energy flux in marine ecosystems, being the only significant consumers of dissolved organic matter (DOM) in the ocean. Generally, about half of the oceanic primary production is channeled through bacterioplankton. They respire most of the DOM taken up and convert the rest into bacterial biomass via growth (del Giorgio and Cole 2000). Bacterial growth in marine environments can be limited by several factors, such as temperature (Pomeroy and Deibel 1986; Kirchman and Rich 1997), DOM availability (Kirchman 1990; Kirchman and Rich 1997), inorganic nutrient availability (Cotner et al. 1997), or micronutrients such as iron (Pakulski et al. 1996; Church et al. 2000).

Large parts of the world's oceans (Equatorial and Subarctic Pacific and the entire Southern Ocean) have been characterized as high-nutrient, low-chlorophyll (HNLC) areas, where the biomass of primary producers is low despite the high concentrations of inorganic nutrients available. There is now convincing evidence from Fe fertilization experi-

ments to indicate that the low Fe concentrations found in HNLC areas are limiting phytoplankton growth (Martin et al. 1991; Coale et al. 1996; Boyd et al. 2000). Despite the importance of bacteria for the carbon and nutrient budgets of the global ocean, bacterial growth limitation by iron in HNLC areas has not received so much attention as phytoplankton production. Iron is an important component of bacterioplankton biomass. The iron to carbon quota of bacterioplankton is about 1.5 to 2 times higher than that of phytoplankton (Maldonado and Price 1999) and bacterioplankton account for 20–45% of the biological Fe uptake in the Subarctic Pacific (Tortell et al. 1996). Additionally, Fe-containing proteins, such as cytochrome *c*, a component of the electron transfer chain in the bacterial respiratory system, play a central role in bacterial metabolism. Fe-depleted bacteria exhibit slow growth and a low growth efficiency (Tortell et al. 1996; Kirchman et al. 2003), which ultimately determines whether bacteria will act in the trophic chain as a “link” (recycling and transferring significant amounts of organic material to higher trophic levels) or as a “sink” (mainly respiring DOM) (Williams 2000).

About 20–40% of the total DOM in the ocean can be characterized as carbohydrates, proteins, and lipids, with higher percentages of macromolecular compounds in the euphotic layers of the ocean compared with deep waters (Benner et al. 1997). Most of the DOM available to bacteria in the ocean comprises compounds >1,000 Da (Amon and Benner 1994, 1996). This high-molecular weight DOM must be hydrolyzed into molecules smaller than about 600 Da before it can be taken up by bacteria (Payne 1980). The hydrolysis of macromolecules might require an important investment in the synthesis of hydrolytic enzymes and the corresponding transport systems to take up the cleavage products (del Giorgio and Cole 2000), and the cost/benefit relationship of extracellular hydrolysis might not always be

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advantageous for an energy-deprived bacterium (Koch 1997). It has been hypothesized that Fe-limited phytoplankton growth could restrict carbon flow to bacteria (Kirchman et al. 2000), resulting in bacteria limited by both labile DOM and Fe (Church et al. 2000). There is little information about the bacterioplankton response to Fe fertilization, but previous studies showed increased bacterial production inside Fe-fertilized waters (Cochlan 2001; Hall and Safi 2001). Changes in the activity pattern of a bacterial assemblage are often related to changes in bacterial community structure (Pinhassi et al. 1997; Arrieta and Herndl 2002) or could be caused by induction of genes repressed under low energy conditions. The purpose of this study was to determine in situ the structural and functional response of a natural bacterioplankton community to Fe fertilization and to decipher the underlying mechanisms causing that response.

## Materials and methods

**Iron fertilization and sampling**—The in situ iron fertilization experiment (EisenEx) took place in the Atlantic sector of the Southern Ocean (~21°E, 48°S) during the cruise ANT XVIII/2 on board the RV *Polarstern* in austral spring (6–29 November 2000). A cyclonic eddy of ~150 km diameter shed by the Antarctic Polar Front was chosen as the experimental site. The center of the eddy was marked with a drifting buoy, and an area of about 50 km<sup>2</sup> around the buoy was enriched with an acidified iron sulfate solution spiked with the tracer SF<sub>6</sub> (Strass et al. 2001). Iron fertilization was repeated twice in 8-d intervals with no further addition of SF<sub>6</sub>. Sampling was performed inside and outside the Fe-enriched “patch” throughout the experiment. The location of the patch was determined prior to the sampling on the basis of underway measurements of SF<sub>6</sub> concentrations in the surface waters (Watson et al. 2001). In-patch stations were chosen on the basis of their high SF<sub>6</sub> concentrations, whereas outside stations showed only background levels of SF<sub>6</sub>. Further SF<sub>6</sub> analyses on discrete water samples taken from the sampling rosette were performed to confirm whether samples came from inside or outside the Fe-enriched water mass (Watson et al. 2001). Samples were collected with a conductivity-temperature-depth (CTD)-rosette (Sea-Bird SBE 32) equipped with 24 bottles (12 liters), CTD probes (Sea-Bird 911 plus), and a Haardt fluorometer. For the bulk measurements of bacterial abundance, production, and ectoenzyme activity, six samples were collected from every station between 10 and 150 m depth. For DNA fingerprinting and zymography, a single large volume of water (150–200 liters) was collected at selected stations at 20–30 m depth.

**Bacterial abundance and production**—Bacterial abundance was estimated by direct counting of formaldehyde-fixed samples (2% final concentration). Bacteria were collected onto 0.02- $\mu$ m pore-size filters (25-mm-diameter Anodisc, Whatman), stained with SYBR Green I, and counted by epifluorescence microscopy (Noble and Fuhrman 1998). Bacterial production rates were estimated from the rate of [<sup>3</sup>H]thymidine and [<sup>3</sup>H]leucine incorporation. Duplicate samples and one formaldehyde-fixed blank containing 10 ml seawater plus 10 nmol L<sup>-1</sup> final concentration of either

tracer were incubated at in situ temperature for 2–5 h. Further processing of the samples followed the methods described elsewhere (Fuhrman and Azam 1982; Kirchman et al. 1985).

**Bulk measurements of bacterial ectoenzymatic activity**—The hydrolysis of the fluorogenic substrate analogs L-leucine-7-amido-4-methylcoumarin, 4-methylumbelliferyl  $\alpha$ -D-glucopyranoside, and 4-methylumbelliferyl  $\beta$ -D-glucopyranoside was measured to estimate the activity of bacterial ectoenzymes of aminopeptidase,  $\alpha$ - and  $\beta$ -glucosidase (Hoppe 1983). Triplicate 5-ml subsamples containing the corresponding substrate at 100  $\mu$ mol L<sup>-1</sup> final concentration were incubated at in situ temperature and measured every 2 h until a significant increase in fluorescence ( $\lambda_{\text{ex}} = 360$  nm,  $\lambda_{\text{em}} = 445$  nm) was detected. When no significant fluorescence increase was measured after 8 h, the ectoenzyme activity was considered to be zero.

**Concentration of bacterial biomass by tangential flow filtration for zymography and DNA fingerprinting**—Large seawater samples (150–200 liters) were filtered through 0.8- $\mu$ m pore-size polycarbonate filters (142 mm diameter, Millipore) to exclude most of the eukaryotic organisms. To minimize clogging, the filter was replaced every 25 liters. Bacteria in the filtrate were concentrated to a final volume of ~0.5 liter with a Pellicon (Millipore) tangential flow filtration system equipped with a 0.2- $\mu$ m pore-size filter cartridge (Durapore, Millipore). Bacteria in the retentate of the Pellicon system were further concentrated by centrifugation (20,000  $\times$  g, 4°C, 30 min). The resulting pellet was washed three times with 0.2- $\mu$ m-filtered seawater and split into aliquots for subsequent analysis (16S rDNA fingerprinting, zymography).

**16S rDNA fingerprinting of bacterial communities**—Terminal-restriction fragment length polymorphism (T-RFLP) analysis of bacterial communities was performed by methods previously described (Moeseneder et al. 2001b). Briefly, DNA was extracted from an aliquot of the bacterial concentrate and subsequently amplified by polymerase chain reaction (PCR) with the use of the Bacteria-specific forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and the universal reverse primer 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). The forward primer (27F) was 5'-labeled with 5-carboxy-fluorescein and the reverse primer (1492R) with 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein. All primers were synthesized by Interactiva. With this technique, two labeled fragments can be obtained from each PCR product after the restriction digest, readily distinguishable by their fluorescence emission wavelength by an ABI Prism 310 capillary sequencer (Moeseneder et al. 2001a). Two restriction enzymes, *Hha*I and *Msp*I, were used independently on each sample to generate the restriction patterns. Only those peaks with a peak area >1% of the total peak area of the electropherograms were counted. Peaks appearing only once in all the samples analyzed were considered outliers and were excluded from further analysis.

**Cloning and sequencing of 16S rDNA fragments**—16S rDNA was amplified by the same primers as for T-RFLP but

without the fluorescent label. The resulting PCR products (~1,500 bp) were cloned with the pMOSBlue blunt-ended cloning kit (Amersham Pharmacia Biotech) following the recommendations of the manufacturer. Plasmids from insert-containing cells were purified by alkaline lysis with sodium dodecyl sulfate and subsequently screened by PCR and T-RFLP as described above. Once the clones were assigned to different terminal restriction fragments, the purified plasmid preparations were sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and ABI 310 capillary sequencer (Applied Biosystems), following the instructions of the manufacturer. Three-cycle sequencing reactions were performed for each clone with the forward primer 27F, the reverse primer 1492R, and an internal primer 518F (5'-CCA GCAGCC GCG GTA AT-3'), yielding three overlapping fragments of ~600 bp, which were assembled to nearly complete sequences of the 16S rRNA gene with AutoAssembler 2.0 software (Applied Biosystems).

**Phylogenetic analysis**—Sequences were checked for chimeras by the Ribosomal Database Project's CHECK\_CHIMERA (Maidak et al. 2001), compared with known sequences with the basic local alignment search tool (BLAST, <http://www.ncbi.nlm.nih.gov/>), and assigned to major bacterial groups by BLAST similarities. Additionally, sequences were aligned to the Hugenholtz alignment of 16S rRNA sequences (<http://rdp.cme.msu.edu/>) with the ARB software package (<http://www.arb-home.de/>) to further validate the results.

**Capillary electrophoresis zymography of bacterial  $\beta$ -glucosidases**—Ectoenzymes were extracted from bacterial concentrates following the method described elsewhere (Arrieta and Herndl 2001, 2002). Aliquots of the bacterial concentrate obtained by tangential flow filtration corresponding to 60 liters of natural seawater were resuspended in 200  $\mu$ l of cold ( $-20^{\circ}\text{C}$ ) extraction buffer containing 40% glycerol (Sigma), 100 mmol  $\text{L}^{-1}$  taurine (Fluka), 20 mmol  $\text{L}^{-1}$  cholic acid (Fluka), and 1 mmol  $\text{L}^{-1}$   $\text{MgSO}_4$ , pH 7.50. Bacterial ectoenzymes were extracted by sonication on ice at 50 W for 30 s and centrifuged again ( $20,000 \times g$ ,  $4^{\circ}\text{C}$ , 60 min). Thereafter, the supernatant containing the enzyme extract was carefully siphoned off and stored at  $-20^{\circ}\text{C}$  for later analysis. Capillary electrophoresis (CE) zymography analysis of  $\beta$ -glucosidase activity was performed using the same setup as described previously (Arrieta and Herndl 2001, 2002), but longer incubation times (30 and 60 min) were required because of the relatively small amount of biomass collected.

## Results

**Description of the study site**—Sampling started immediately before the first Fe release (7 Nov 00, day 0) and covered a period of more than 3 weeks until day 22 (29 Nov 02). During this period, day length increased from 15 to 16 h, water temperature increased by about  $1^{\circ}\text{C}$  (from  $3.5^{\circ}\text{C}$  to  $4.2^{\circ}\text{C}$  in surface waters and from  $2.0^{\circ}\text{C}$  to  $3.5^{\circ}\text{C}$  at 100 m depth). Chlorophyll *a* (Chl *a*) concentrations increased about fourfold inside the Fe-fertilized patch compared with the outside stations (Gervais et al. 2002). Inorganic nutrient con-

centrations remained high over the whole study period both inside and outside the patch. The average concentration over the first 100 m of the water column exceeded  $20 \mu\text{mol L}^{-1}$  nitrate and  $1.5 \mu\text{mol L}^{-1}$  phosphate at all stations. The depth and shape of the Fe-fertilized patch varied considerably during the experiment. It was initially confined to the first 40 m of the water column, but after heavy storms from day 5 to 8 and again from day 12 to 15, the  $\text{SF}_6$  signature of the Fe-fertilized patch was detectable down to 80 m depth. The in-patch stations at days 11 and 17 were situated on the edge of the patch, as indicated by the lower  $\text{SF}_6$  concentrations (Watson et al. 2001). Although the bulk parameters measured at these stations agree with the general picture of stimulation of bacterial activity found for the Fe-fertilized waters (Fig. 1), these stations are not considered to be true in-stations following a previous publication (Gervais et al. 2002). Only the enzyme diversity samples, which were collected from a single depth in a separate cast, are included for these days.

**Bacterial abundance and production**—Bacterial abundance in the first 40 m of the water column ranged from  $0.20$  to  $0.62 \times 10^9$  bacteria  $\text{L}^{-1}$  inside the Fe-fertilized patch and from  $0.25$  to  $0.44 \times 10^9$  bacteria  $\text{L}^{-1}$  outside the patch (Figs. 1a, 2a). The evolution of the bacterial abundance inside the Fe-fertilized water mass revealed a consistent increase from  $\sim 0.3$  to  $\sim 0.6 \times 10^9$  bacteria  $\text{L}^{-1}$  over the first 12 d (Fig. 2a). This higher bacterial abundance inside the patch was maintained until the end of the experiment, while no comparable increase in bacterial abundance was observed outside the Fe-fertilized patch.

Leucine incorporation, a measure of protein synthesis, ranged from  $6.60$  to  $25.78$  pmol leucine  $\text{L}^{-1} \text{h}^{-1}$  inside the patch and from  $6.55$  to  $14.47$  pmol leucine  $\text{L}^{-1} \text{h}^{-1}$  in the surrounding waters (Figs. 1b, 2b). Thymidine incorporation ranged from  $0.51$  to  $2.96$  pmol thymidine  $\text{L}^{-1} \text{h}^{-1}$  in the upper 40 m of the water column inside the patch and from  $0.062$  to  $1.016$  pmol thymidine  $\text{L}^{-1} \text{h}^{-1}$  in nonfertilized waters (Figs. 1c, 2c). Leucine and thymidine incorporation in the Fe-fertilized patch peaked a few days after each of the successive iron additions (Fig. 2b,c) and rapidly decreased to the background levels observed outside the patch, despite the increase in bacterial abundance. This tendency was also reflected by the cell-specific incorporation rates (Fig. 2d,e), although not so pronounced in the case of cell-specific leucine uptake.

**Bacterial ectoenzyme activity**—Bacterial hydrolytic activity at saturating concentrations of substrate measures the amount of enzyme present rather than the actual hydrolysis rates in the sample. Three enzyme activities were measured: two involved in the hydrolysis of carbohydrates ( $\alpha$ - and  $\beta$ -glucosidase) and one responsible for the hydrolysis of proteinaceous material (leucine-aminopeptidase). The ectoenzymes cleaving carbohydrates,  $\alpha$ - and  $\beta$ -glucosidase (Figs. 1d,e, 3a,b), showed a consistently higher activity inside the Fe-fertilized patch after day 5 than outside, increasing from almost undetectable levels to a maximum of  $0.38$  and  $0.59$  nmol  $\text{L}^{-1} \text{h}^{-1}$  for  $\alpha$ - and  $\beta$ -glucosidase, respectively. Cell-specific  $\alpha$ - and  $\beta$ -glucosidase activities (Fig. 3d,e) showed a

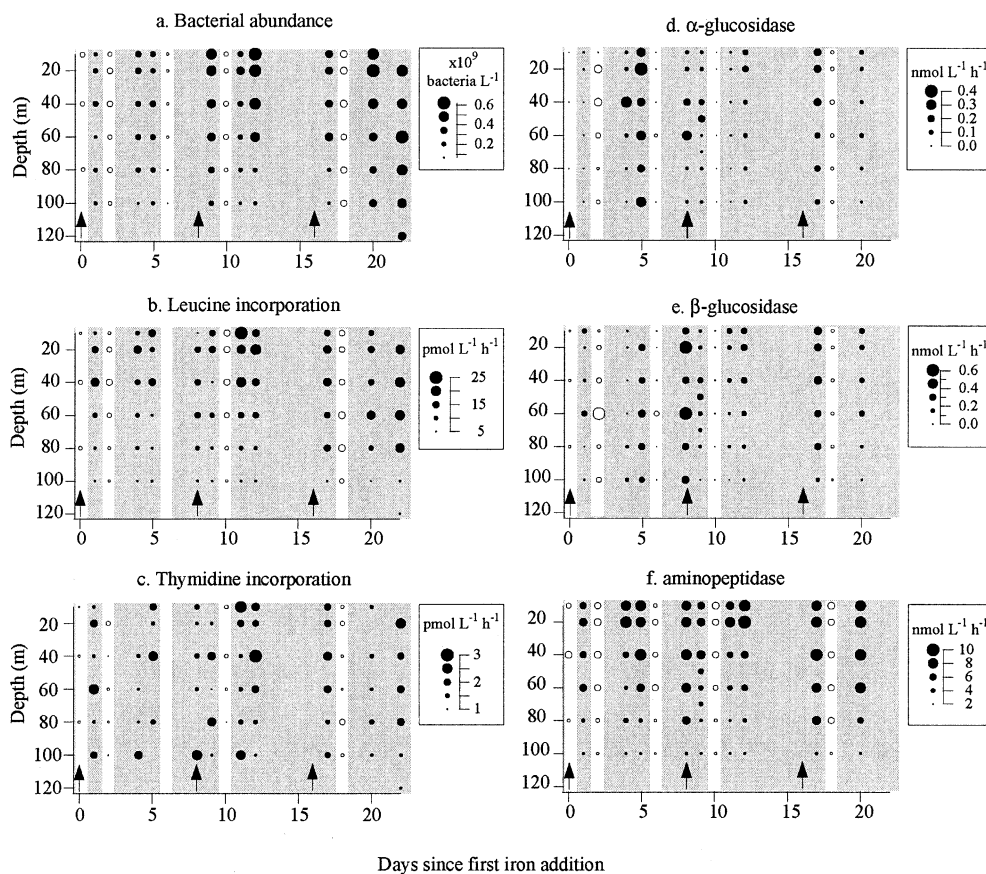


Fig. 1. Overview of the dynamics of (a) bacterial abundance, (b) leucine incorporation, (c) thymidine incorporation and bacterial ectoenzymatic activities, (d)  $\alpha$ -glucosidase, (e)  $\beta$ -glucosidase, and (f) aminopeptidase at different depths. Symbols indicate samples taken inside (solid circles, gray background) or outside (open circles, white background) of the Fe-enriched patch. The arrows at the bottom of each panel indicate the days of Fe release.

similar pattern, with higher values inside the patch (Mann–Whitney  $U$ -test,  $P < 0.05$ ) than outside the patch. Aminopeptidase activity (Figs. 1f, 3c) was overall about two orders of magnitude higher than  $\alpha$ - and  $\beta$ -glucosidase. Inside the patch, aminopeptidase activity showed a considerable increase, ranging from 4.13 to 9.32  $\text{nmol L}^{-1} \text{h}^{-1}$ , while outside the patch, aminopeptidase varied from 2.81 to 5.47  $\text{nmol L}^{-1} \text{h}^{-1}$ . After day 5, the bulk aminopeptidase activity inside the Fe-fertilized patch was almost twice as high as outside the patch, matching the increase in bacterial abundance and resulting in no significant increase (Mann–Whitney  $U$ -test,  $P > 0.05$ ) in cell-specific aminopeptidase activity (Fig. 3f).

**Bacterial species richness and phylogeny**—T-RFLP analysis consistently yielded the same 19 operational taxonomic units (OTUs) for the forward primer (Fig. 4) and 13 OTUs for the reverse primer in all the samples analyzed from inside and outside the Fe-fertilized patch with the use of *HhaI*. Digestion with *MspI* resulted in 17 OTUs for the forward primer and 12 OTUs for the reverse primer. As with the restriction enzyme *HhaI*, no differences in the patterns of presence and absence of the different OTUs between inside the Fe-fertilized patch and outside the patch were found with

*MspI*. Because the number of OTUs detected with *HhaI* was slightly higher, this restriction enzyme was used for screening the clone libraries. Two clone libraries were made in order to maximize the chance of finding different species potentially hidden in the same T-RFLP fragment: one from an outside station sampled at day 10 and the other from an inside station at day 20. The inserts contained in 50 randomly picked clones from each sample were screened by T-RFLP (both forward and reverse primers labeled) and sequenced if found to be different. In total, 22 clones were sequenced yielding 12 bacterial sequences (GenBank accession numbers AY135663–AY135675) plus 9 sequences clustering with algal plastids (GenBank accession numbers AY135676–AY135684) and 1 chimeric sequence. Only 10 out of the 19 OTUs obtained by digestion with *HhaI* could be identified following this approach (Fig. 4), but most of the largest peaks were represented in the clone library. The phylogenetic affiliation of the sequenced OTUs is shown in Fig. 5. The additional data on the forward and reverse restriction fragments of single clones obtained during screening did not show any differences between the bacteria inhabiting the Fe-fertilized patch and the surrounding waters that could have been masked in the community fingerprinting. PCR reactions with primers specific for Archaea, as described in Moese-

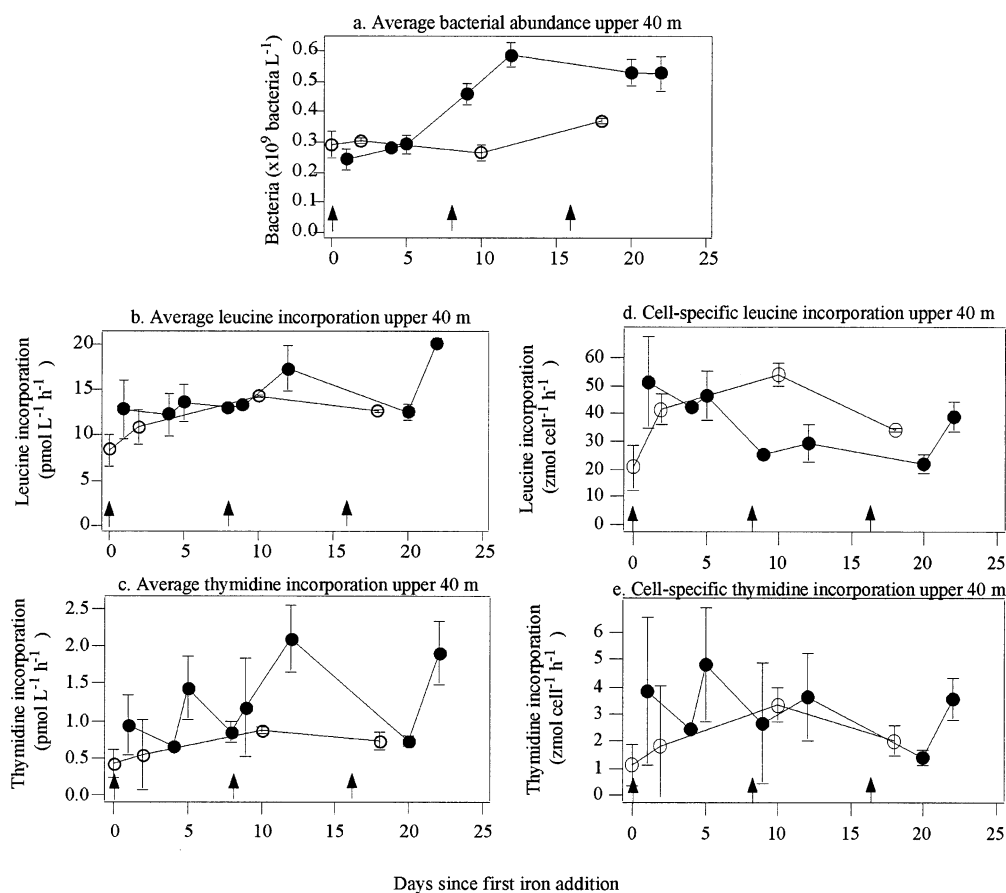


Fig. 2. Average values ( $\pm$ SD) of (a) bacterial abundance, (b) leucine and (c) thymidine incorporation, (d) cell-specific leucine incorporation, and (e) cell-specific thymidine incorporation in the upper 40 m of the water column. Symbols indicate samples taken inside (solid circles) or outside (open circles) of the Fe-enriched patch. The arrows at the bottom of each panel indicate the days of Fe release. ( $zmol = 10^{-21}$  mol)

neder et al. (2001a), did not yield detectable amounts of PCR product, probably because of the relatively low abundance of Archaea in surface waters.

**Diversity of bacterial  $\beta$ -glucosidases by CE zymography**—The fingerprints of  $\beta$ -glucosidase activity showed a total of 10 different  $\beta$ -glucosidases detectable in either Fe-fertilized waters or outside the patch. The zymography fingerprints are shown in Fig. 6. There were remarkable differences between the fingerprints obtained inside the Fe-fertilized patch and outside the patch after day 9. Corresponding enzymes showed much higher levels of activity inside than outside the patch, and three additional enzymes (marked with circles in Fig. 6) appeared inside the Fe-fertilized waters that were not detectable outside the Fe-fertilized patch. These three additional  $\beta$ -glucosidases, not detected outside the patch, comprised about 55% of the total  $\beta$ -glucosidase activity. Enzyme 4, however, reached its maximum activity in the patch on day 17 and then declined progressively, as can be seen in the electropherograms of days 20 and 22. As an exception to this rule, the out-station of day 18 showed a small amount of  $\beta$ -glucosidases 5 and 6 not detectable at other out-stations. However, these small

peaks made up only about 16% of the total  $\beta$ -glucosidase activity detected at that station and their total intensity was much lower than that of the corresponding peaks detected inside the patch (Fig. 6).

## Discussion

Considerable variation between the bacterial activities was measured at the out-stations (Figs. 1–3). Because the out-stations were situated anywhere around the Fe-fertilized patch but not in a distinct water mass, bacterial activities measured at the out-stations probably do not represent a specific trend in the development of the unfertilized water mass, but rather the heterogeneity of the surrounding waters. Despite this variability, our data show that bacteria inhabiting the Fe-fertilized waters roughly doubled their abundance within 12 d, whereas bacterial abundance outside the patch remained essentially constant (Fig. 2a). This fact alone proves the positive effect of Fe fertilization on bacterioplankton. Similar increases in bacterial abundance have been reported previously in Fe-amended bottle experiments (Pakulski et al. 1996; Hutchins et al. 1998; Kirchman et al. 2000), whereas others (Church et al. 2000) found that Fe alone had

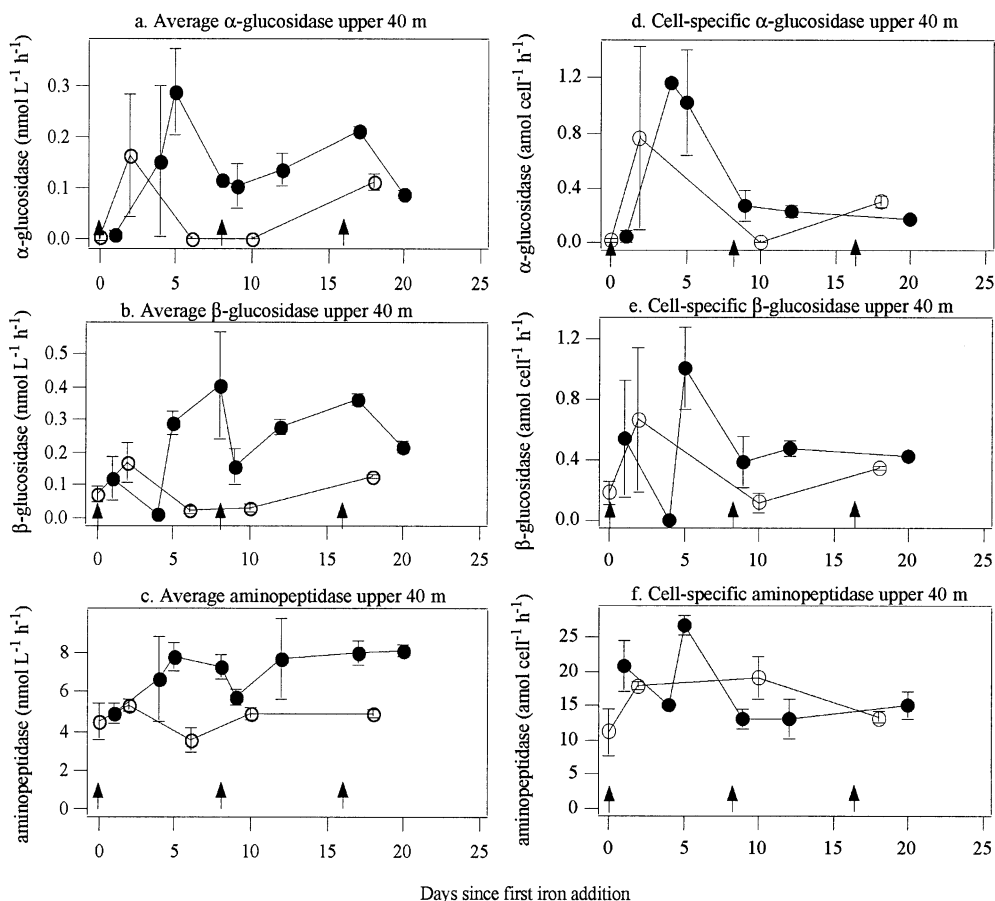


Fig. 3. Average bulk values and cell-specific hydrolysis rates ( $\pm$ SD) of the different ectoenzymes (a, d)  $\alpha$ -glucosidase, (b, e)  $\beta$ -glucosidase, and (c, f) aminopeptidase in the upper 40 m of the water column. Symbols indicate samples taken inside (solid circles) or outside (open circles) of the Fe-enriched patch. The arrows at the bottom of each panel indicate the days of Fe release. (amol =  $10^{-18}$  mol)

little effect on bacterial growth. The results of previous mesoscale Fe fertilization experiments are contradictory. An increase in bacterial abundance similar (1.7-fold) to the one reported here was observed during IronEx II (Cochlan 2001), whereas grazing pressure prevented an increase in bacterial abundance during SOIREE (Hall and Safi 2001). No data are available on grazing on bacterioplankton during EisenEx, but the abundance and biomass of heterotrophic nanoflagellates doubled inside the patch compared with unfertilized waters (Verity unpubl. data). Although increased grazer abundance probably had a significant effect on bacterioplankton growth during EisenEx, the control was not as stringent as during SOIREE.

There was a certain delay in the reaction of bacterioplankton to the successive Fe fertilizations. Both leucine and thymidine incorporation rates showed peaks in bacterial production a few days after each fertilization event (fertilization at days 0, 8, and 16; peaks in production at days 5, 12, and 22). Thereafter, bacterial production declined again to levels comparable to those found outside the patch. This pattern is in contrast to the more continuous increase in bacterial production observed during IronEx II, in which the increase in bacterial production correlated with the increase in Chl *a*

(Cochlan 2001), and during SOIREE, in which an increase in bacterial production was observed only at the end of the experiment (Hall and Safi 2001). Although the EisenEx study was considerably longer (22 d) than IronEx II (14 d) and SOIREE (13 d), the time between the successive Fe fertilizations was longer in EisenEx (8 d) than in IronEx II (3–4 d) and SOIREE (2–4 d). This could have allowed bacteria to respond to the decrease in dissolved Fe in between fertilizations. If true, this would point to direct iron limitation of bacterial growth.

However, the current view is that, at least in some HNLC areas, bacterioplankton are not directly Fe limited, but rather carbon-limited as a consequence of the low primary productivity of Fe-limited phytoplankton (Hutchins et al. 1998; Church et al. 2000; Kirchman et al. 2000). Primary productivity, however, did not show such a discontinuous pattern inside the patch. Phytoplankton production slowly increased from day 2 onward. At day 4, a steep increase in phytoplankton production was recorded, maintaining high productivity levels until day 16. Enhanced phytoplankton growth in the patch was observed until the end of the experiment (Gervais et al. 2002). Although we do not have data on the release of freshly produced dissolved organic carbon by phy-

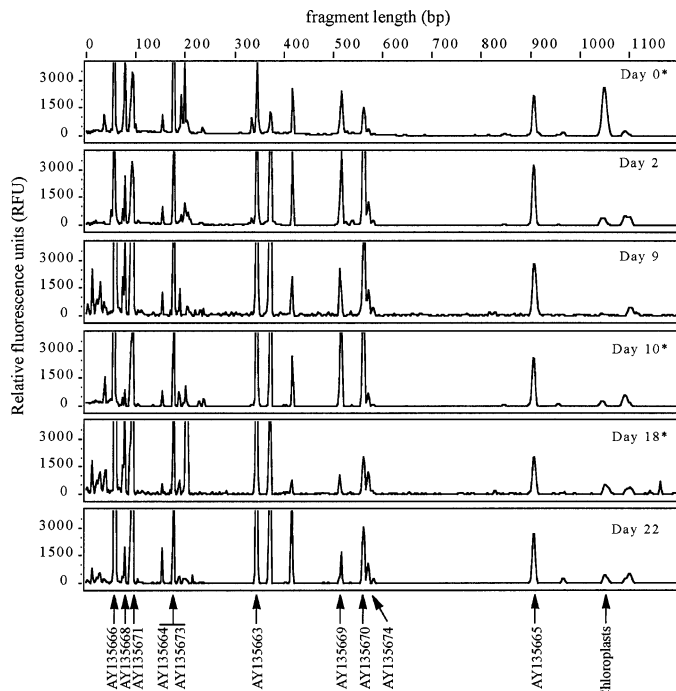


Fig. 4. T-RFLP fingerprinting (forward primer, *HhaI*) of the microbial communities at different time points of this study. The numbers in the upper right corner indicate the number of days after the initial Fe release; an asterisk indicates samples taken outside of the patch. The GenBank accession numbers of the cloned sequences corresponding to each peak are given at the bottom if available.

toplankton, it seems unlikely that dissolved primary production occurred in pulses given the rather constant increase in particulate primary production from day 4 until day 16. In fact, bacterial production seemed to be rather uncoupled from the dynamics of particulate primary production. One possible explanation for these discrepancies between our findings and those reported from bottle experiments is that during in situ fertilization, iron is not contained in a bottle, and because of its low solubility in seawater, it can sink out of the water column quite rapidly. Bacteria in our system were exposed to short pulses of iron availability similarly to natural fertilization events that occur, for example, when icebergs release iron as they melt (Sedwick and DiTullio 1997). It could well be that bacterioplankton growth was limited by carbon in the beginning, but once the carbon supply was granted by permanently enhanced primary production, the effects of an intermittent increase in dissolved Fe would become more apparent, leading to the observed peaks in bacterial production.

Although bacteria inside the Fe-fertilized patch were growing faster at some points than outside the patch, no significant differences in thymidine uptake were found between the bacteria inside and outside the patch. The cell-specific leucine uptake ratio shows a slight tendency to decrease in Fe-fertilized waters, leading also to a decrease in the leucine to thymidine uptake ratio (data not shown). However, the observed values fall within the range observed also in waters outside the patch (Mann-Whitney *U*-test,  $P > 0.05$ ). These data suggest that the basic physiological prop-

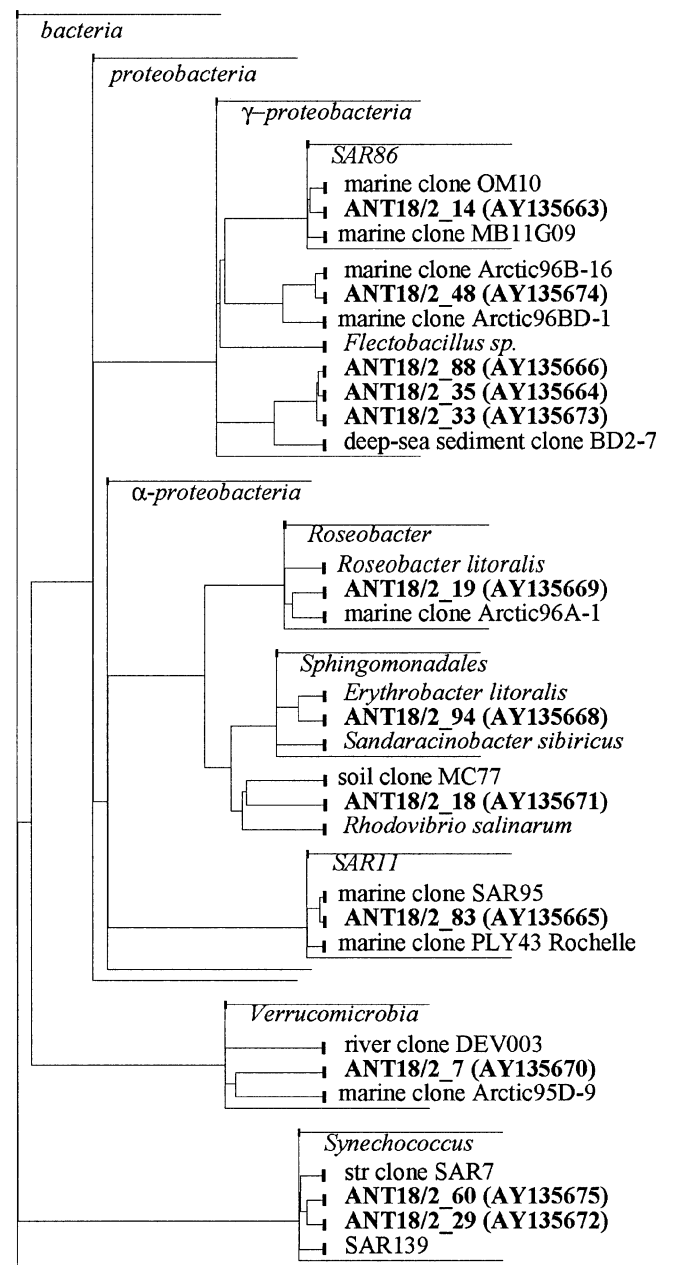


Fig. 5. Cladogram indicating the affiliation of the different bacterial OTUs sequenced (in bold). GenBank accession number indicated in parentheses.

erties of the bacterioplankton community were not significantly altered by Fe fertilization.

Bacterial ectoenzyme production constitutes a substantial investment, and bacterial communities often modulate their hydrolytic activities by reducing the synthesis of hydrolases in the presence of the corresponding low-molecular weight product or enhance enzyme production when significant concentrations of polymeric material are present (Chróst 1991). Specific  $\alpha$ - and  $\beta$ -glucosidase activity increased considerably after day 5 from barely detectable in most out-stations to substantial average rates of about  $0.15 \text{ nmol L}^{-1} \text{ h}^{-1}$  for  $\alpha$ -glucosidase activity and  $0.3 \text{ nmol L}^{-1} \text{ h}^{-1}$  for  $\beta$ -glucosi-



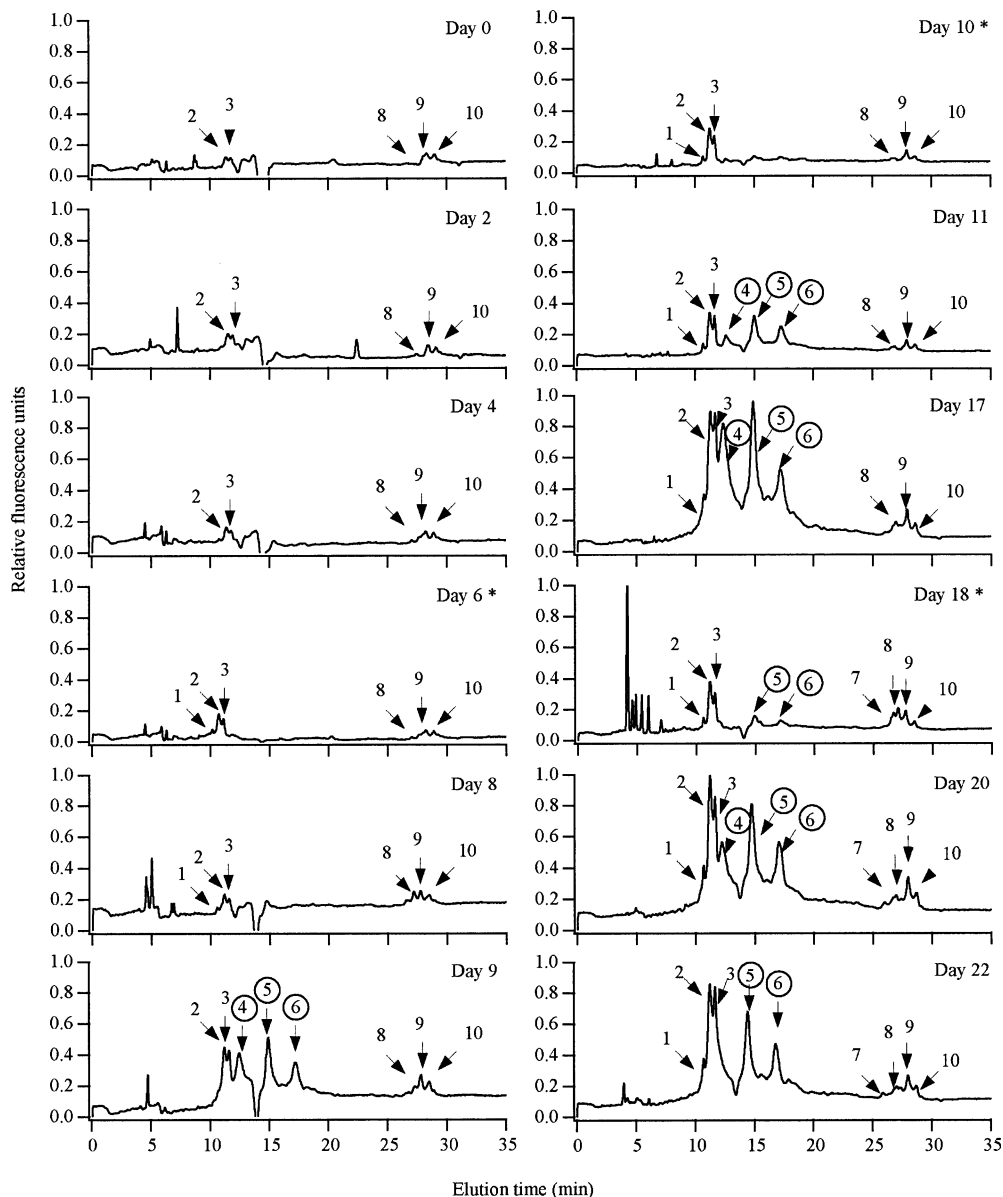


Fig. 6. Profiles of  $\beta$ -glucosidase activity expressed by the bacterial community at different times of the experiment. The time in days after the first Fe release is given in the upper right corner of the profile. An asterisk after the day number indicates that the sample was collected outside the Fe-enriched patch. Arrows indicate the different  $\beta$ -glucosidases found in each sample. Numbers indicate the identity of peaks matching corresponding peaks in different samples. The circled numbers indicate newly appearing enzymes as described in the text.

dase (Figs. 1d,e, 3a,b). Because dissolved carbohydrate concentrations were not measured during the cruise, we do not know whether dissolved carbohydrate release was enhanced after Fe fertilization or whether dissolved carbohydrates could not be hydrolyzed by the Fe-limited community. Although  $\alpha$ - and  $\beta$ -glucosidase activities showed large variations outside the patch, both the bulk values and the cell-specific hydrolysis rates were significantly higher for the in-patch samples compared with the surrounding waters (Mann-Whitney  $U$ -test,  $P < 0.05$ ). In contrast to  $\alpha$ - and  $\beta$ -glucosidase, aminopeptidase activity increased along with bacterial abundance, both roughly by a factor of two inside

the patch (Figs. 2a, 3c) within the first 12 d of the experiment. Cell-specific aminopeptidase activity (Fig. 3f) did not show significant differences between the bacteria in the Fe-fertilized area and those in the surrounding waters (Mann-Whitney  $U$ -test,  $P > 0.05$ ).

Whether bacteria are directly or indirectly limited by iron availability, they efficiently compete with phytoplankton for Fe acquisition. Size fractionation experiments have shown that bacteria can be responsible for up to 50–70% of the total Fe uptake (Tortell et al. 1996; Maldonado and Price 1999). Bacteria can produce siderophores to capture Fe at low concentrations, and those siderophores can be used by

species different from the one that produced them (Granger and Price 1999). Thus, one would assume that some bacterial species are better competitors than others, resulting in a certain pattern of species dominance that could change, once Fe is no longer limiting. Our data showed, however, no evidence of an Fe-induced shift in the bacterial community structure. The T-RFLP patterns remained basically unchanged both inside and outside of the Fe-fertilized water mass, indicating no change in the species richness. These results agree with previous studies in seawater cultures (Hutchins et al. 2001) showing that iron addition had little or no effect on bacterial community composition over a short period of time (4–5 d). There could have been more subtle changes in the evenness (the relative abundance of each species) not accounted for by our analysis of presence or absence of OTUs. Hutchins et al. (2001) used the peak intensity of the T-RFLP patterns as an estimate of evenness. Such analysis is subjected to potential bias caused by electrokinetic injection when a capillary sequencer is used and by the PCR amplification of heterogeneous mixtures of templates (Suzuki and Giovannoni 1996), limiting the quantitative interpretation of the results. Although relative peak height varies from sample to sample, there is no identifiable trend in the variations in T-RFLP peak intensity in samples collected inside the Fe-fertilized patch (Fig. 4) that would indicate a major shift in species dominance. This lack of changes detected over the 22-d period, together with the lack of spatial heterogeneity in the relatively large area sampled, suggests that bacterial communities in this part of the Southern Ocean are well adapted to their environment and that the community structure is remarkably stable. The phytoplankton community, in contrast, showed considerable differences in species composition between the Fe-fertilized patch and the surrounding waters (Assmy and Henjes unpubl. data). Although iron alone does not seem to change bacterial community structure, the possibility of a subsequent indirect effect cannot be excluded. The collapse of the observed Fe-induced phytoplankton bloom and the following release of significant amounts of DOM could trigger such a change in the bacterial community structure.

This apparent stability of the bacterial community contrasts with the dramatic changes observed in the patterns of  $\beta$ -glucosidase expression determined by capillary electrophoresis zymography (Fig. 6). The  $\beta$ -glucosidases found outside the Fe-fertilized patch exhibited much higher activity levels inside the patch; moreover, three new  $\beta$ -glucosidases that were hardly or not at all detectable in the stations surrounding the Fe-enriched patch were expressed in large amounts inside the patch (Fig. 6). These results obtained from  $\beta$ -glucosidase fingerprinting clearly show that bacteria not only grew faster inside than outside the Fe-enriched patch, but they also activated genes that were silent at the beginning of the experiment. However, no changes were detected in the phylogenetic composition of the bacterial community that could explain these large shifts in  $\beta$ -glucosidase expression. Thus, the phenotype rather than the structure of the bacterial community was altered by iron addition.

Although no changes were detectable in the phylogenetic composition of the bacterial community in relation to Fe fertilization, the heterotrophic activity of the bacterial com-

munity changed dramatically, as shown by the net accumulation of bacterial biomass and the activation of the carbohydrate-degrading ectoenzymes  $\alpha$ - and  $\beta$ -glucosidase in the bulk measurements. Further evidence is provided by the induction of new enzymes not present prior to Fe fertilization shown by the  $\beta$ -glucosidase zymography (Fig. 6). The results presented here are in contrast to previous observations in which we found the induction of  $\beta$ -glucosidase activity linked to major changes in the community structure during the wax and wane of a coastal phytoplankton bloom (Arrieta and Herndl 2002). Despite the obvious differences between the HNLC site studied here and the coastal North Sea and the shorter time span of this study, our data suggest that bacterial communities in the Southern Polar Frontal Zone are remarkably stable and adapted to episodic iron inputs. Regardless of whether bacteria are limited by iron directly or indirectly, the results reported here confirm that bacterial activity is not only quantitatively enhanced by Fe fertilization but also qualitatively altered as indicated by CE zymography. However, it is important to stress that these results only cover the initial response of the bacterioplankton community to Fe fertilization and that the longer term effects of prolonged Fe fertilization, enhanced primary production, and the eventual collapse of the phytoplankton bloom remain to be investigated.

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